

Review article

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INVOLVEMENT OF microRNAs IN THE INFLAMMATORY PATHWAYS OF PULMONARY SARCOIDOSIS

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Sarcoidosis is a multi-organ disease in which affected tissues are invaded with non-necrotizing granulomatous structures, mostly consisted of T helper 1 (Th1) cells and multinucleate giant cells. However, the etiology and pathogenesis of sarcoidosis is not known and the diagnosis is usually based on clinical examination involving radiography and histopathological analysis of biopsies of affected organs. Although the knowledge on the molecular background of sarcoidosis is limited, it seems that the important pathways involve transforming growth factor- β (TGF- β) and JAK/STAT, which may influence the interferon- γ (IFN- γ)-mediated signaling. Additionally, recently the role of microRNAs (miRNAs), the small non-coding RNA molecules, has been emphasized in different pathological conditions including autoimmune diseases. This review summarizes the current knowledge on the molecular pathways in the pathogenesis of sarcoidosis with a special emphasis on cytokines and miRNAs controlling immune cells proliferation and differentiation. Moreover, the possible role of T regulatory cells (CD4⁺ CD25⁺ FoxP3⁺) in this disease has been discussed.

Key words: sarcoidosis, interferon- γ , immune response, miRNA, Treg, transforming growth factor- β , helper T cell type 1, Toll-like receptor 2

INTRODUCTION

Sarcoidosis (also known as Besnier-Boeck-Schaumann disease) is a chronic, multi-organ disorder of unknown etiology. It is characterized by formation of non-necrotizing granulomas in different organs, what leads to their damage or dysfunction. In more than 90% of cases systemic sarcoidosis affects lungs and mediastinal lymph nodes. Extrapulmonary involvement usually concerns skin, peripheral lymph nodes, salivary glands, eyes, central and peripheral nervous system. Granulomas' formation in the bone marrow leads to severe hematological disorders, while cardiac involvement affecting up to 30% of sarcoidosis patients can lead to severe cardiac complications (conduction abnormalities, ventricular arrhythmias, progressive heart failure) (1). Unfortunately, cardiac involvement in patients with sarcoidosis is difficult to diagnose. Therefore, the physicians must keep a high index of suspicion in order to immediately introduce the life-saving treatment. The disease most often occurs in adults aged 25 to 40 years old. Symptoms depend on the affected organ and the most common are shortness of breath, cough, fatigue, fever, pain in the joints, erythema nodosum, anergy and - in the advanced stage of pulmonary involvement - sarcoidosis-associated pulmonary hypertension (SAPH) (2-4). The course of the disease can be either acute or chronic.

Since no single agent responsible for the development of the disease was characterized so far, diagnosis of sarcoidosis is based on medical examination and exclusion of other diseases.

Chest x-ray pictures may reveal sarcoid granulomas in the lungs and enlarged chest lymph nodes. Further histopathological analysis, high-resolution computed tomography (HRCT), [18F] fluorodeoxyglucose positron emission tomography (FDG-PET) or magnetic resonance imaging (MRI) can be useful to confirm the disease (4, 5). The most important step is the biopsy of invaded organs. As it is crucial to distinguish sarcoidosis from other granulomatous diseases like lymphoma, tuberculosis and tumors, non-necrotizing granulomas should be identified in more than one organ with concomitant exclusion of any other diseases (2, 3). Modern strategy for diagnosis of mediastinal lymphadenopathy involves less invasive procedures than in past years. Endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) is a key diagnostic tool to obtain cytological specimens from enlarged mediastinal lymph nodes (6-8). Endobronchial biopsy of mucosa and transbronchial lung biopsy provide additional diagnostic yield to EBUS-TBNA. Another very common and useful procedure is bronchoalveolar lavage (BAL) fluid investigation. Evaluation of a percentage of lymphocytes and CD4/CD8 ratio in BAL fluid, as well as the level of different biomarkers in BAL fluid and serum such as neopterin, soluble IL-2 receptor, tumor necrosis factor- α (TNF- α), C-reactive protein (CRP), monocyte chemotactic protein (MCP-1), macrophage inflammatory protein 1 α (MIP-1 α) or angiotensin-converting enzyme (ACE) may help to distinguish sarcoidosis from other diseases including non-

sarcoidosis interstitial lung disease (2, 3). However, due to low specificity and sensitivity, measurement of concentration of the abovementioned molecules in serum does not prove to be an effective method for confirmation of the disease and has little clinical significance (5). Also, the level of these factors as well as other cytokines and chemokines could vary significantly depending on ethnic background (Caucasian, African-American and Asian) (3, 9, 10), immunological and disease status. Finally, expert clinician assessment is needed to reach a final diagnosis.

MOLECULAR PLAYERS IN GRANULOMA FORMATION

The non-necrotizing granulomas, appearing in numerous organs, consist mostly of tissue macrophages, CD4⁺ T helper 1 (Th1) lymphocytes and multinucleate giant cells (4). The giant cell is made up of many macrophages that fuse together and is seen in the central region of the granuloma. Study of neuromuscular sarcoidosis shows that tissue macrophages are polarized into M2 phenotype (11). In the early phase of immune response follicular hyperplasia and sinus histiocytosis are similar to the symptoms of non-specific granulomatous

lymphadenitis (12). Later, small epithelioid nodules appear within the node. In the peak phase the granulomas are clearly visible in the lymph nodes while in the late phase the collagen fibers may be produced, resulting in granulomas fibrosis and hyalinization (12, 13).

Moller and Chen suggested that the key element in the pathogenesis of sarcoidosis is the interaction between major histocompatibility complex - MHC (alias human leukocyte antigen - HLA) class II on antigen-presenting cell (APC), antigen and T-cell receptor (TCR) on CD4⁺ T cell (14) (Fig. 1). This provides the first activation signal for antigen-specific T cells. The second signal provided by costimulatory molecules stimulates T cells to produce cytokines that orchestrate immune responses leading to granuloma formation.

According to that, the HLA class II gene alleles strongly determine the clinical outcome, such as progression and severity of the disease. Many other groups have also indicated that some of the alleles of HLA genes may increase the risk of developing the disease (9, 10, 15, 16). Genome-wide analysis by Shurmann *et al.* has shown that loci heterogeneity in MHC region as well as in some minor genes located on chromosomes 1, 3, 7, 9 and X are associated with susceptibility to sarcoidosis (17). Others have suggested different genome regions as potentially

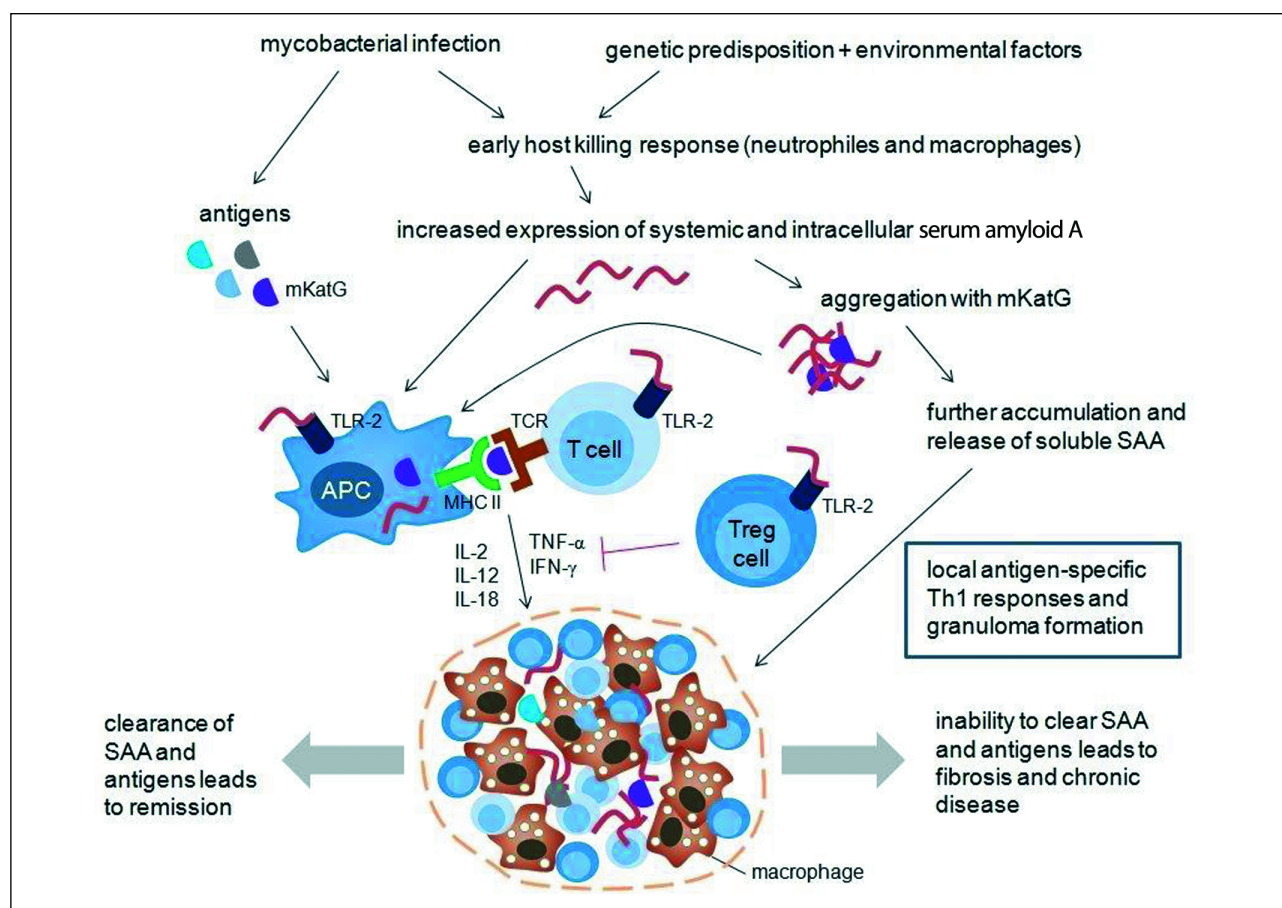


Fig. 1. Hypothetical model of the immunopathogenesis of sarcoidosis. Mycobacterial antigens released upon mycobacterial infection induce the macrophage- and neutrophil-dependent innate immune response. Host proteins (particularly serum amyloid A, SAA) induced as part of this response aggregate with a mycobacterial protein catalase peroxidase (mKatG). Macrophages and dendritic cells (antigen-presenting cells, APC), activated by mKatG, host protein SSA and their aggregates, produce the helper T cell type 1 (Th1)-promoting cytokines IL-2, IL-12, IL-18, TNF- α , and IFN- γ . Antigen-specific Th1 responses orchestrate the complex process of granuloma formation and accompanying inflammation. Removal of mKatG, SAA and mKatG-SAA aggregates results in granuloma regression and disease remission. Failure to remove them results in persistent inflammation and chronic disease.

significant in the disease etiology, among which: 5p, 5q, 12p, 11p, 20q, 9q, 1p and 2p were identified (18, 19). A significant association was reported in German population between sarcoidosis and polymorphism in the butyrophilin-like 2 (BTNL2) gene (rs2076530 A allele) (20). BTNL2 is a member of the immunoglobulin gene family that functions to inhibit T cell activation (21).

Additionally, it has been recently discovered that serum amyloid A (SAA) protein regulates granulomatous inflammation through Toll-like receptor 2 (TLR-2) (22) (Fig. 1). TLR-2 is a membrane protein that is expressed on the surface of phagocytic cells: microglia, Schwann cells, monocytes, macrophages, dendritic cells (DC) as well as T and B cells. Interaction between SAA protein and TLR-2 initiates cytokine release, intensifying the immune response. It has also been proven that the level of that serum protein may indicate disease progression (23). Other publications report on the significance of TLR-9 in pulmonary sarcoidosis (24, 25). The involvement of TLR-9 indicate the recognition of intracellular patterns, which is characteristic for autoimmune diseases. On the other hand, *Mycobacterium tuberculosis*, a putative infectious agent linked to sarcoidosis pathogenesis (Fig. 1), can activate TLR-9. It was shown that mice lacking both TLR9 and TLR2 were significantly more susceptible to *M. tuberculosis* infection than mice with single deficiency of either TLR2 or TLR9 (26). This indicates a significant role for TLR9 and its cooperation with TLR2 in host defense to mycobacterial infection.

IMMUNE CELLS INTERPLAY IN SARCOID GRANULOMA

Granulomas consist of two distinguishable layers: an epithelioid core and surrounding, peripheral layer of infiltrated lymphocytes - in which there are CD4⁺ and CD8⁺ cells, regulatory T lymphocytes, B lymphocytes, macrophages and DCs (27, 28). The importance of DCs in the generation of sarcoid granulomas has been pointed out in several studies. These cells act as professional APCs with the ability to influence T cell activation and differentiation. While, based on flow cytometry, myeloid DCs are just about 1% of all BAL fluid cells, during inflammation their number significantly increases in lung alveolar space compared to alveolar macrophages (29). Importantly, DCs in sarcoidosis do not mature in the lung and skin, but they do mature in the lymph node (29). Mature DCs possess the ability to activate both naive and effector T cells (30). TNF- α produced by inflammatory DCs encourages CD4⁺ T cell proliferation and survival, both indirectly by inducing DCs maturation into APC and directly through the induction of the T cell IL-2 receptor (IL-2R) (30). Many research groups have described the significance of TNF- α within granulomas (31-33).

Shifting the balance of Th cells into Th1 phenotype in sarcoidosis leads to increased production of IFN- γ , IL-2, IL-12, IL-18, and TNF- α within the granuloma (29) (Fig. 1). Th1 character of the granuloma-infiltrating T-lymphocytes was additionally confirmed by bioinformatic analysis, as the signaling network associated with sarcoidosis, was found to be regulated by signal transducer and activator of transcription-1 (STAT1) - the molecule located downstream of IFN- γ receptor in cell signaling pathways (34, 35). However, it is still unclear how this phenomenon contributes to the formation of these non-necrotizing structures. Possibly, interaction between Th1 cells and another T lymphocyte subpopulation (Th17) that invade granulomas is the key factor for their maintenance (36, 37).

Other cells, which seem to play an important role in the prolonged inflammation and granuloma formation in

sarcoidosis, are T regulatory (Treg) lymphocytes. These cells, defined as CD4⁺CD25⁺FoxP3⁺ cells, accumulate at the periphery of the sarcoid granuloma (Fig. 1), are present in the BAL fluid and peripheral blood of sarcoidosis patients. It has been demonstrated that Treg cells from sarcoidosis patients inhibit proliferation of effector T lymphocytes independently of their origin (peripheral blood, BAL fluid or lymph nodes) (28, 38). This antiproliferative activity of Treg cells may be the cause of peripheral CD4⁺ T helper cells anergy noted in sarcoidosis. Majority of Treg cells was found in patient's lymph nodes, where the T lymphocytes mature. These cells may be also responsible for the sustaining of granulomas' formation (39). What is more, different effect of Treg cells on inflammatory cytokine secretion by effector T lymphocytes was demonstrated in healthy people and patients with sarcoidosis (28). Unlike Treg cells from healthy individuals, sarcoidosis regulatory lymphocytes do not completely inhibit IFN- γ and TNF- α production by effector T cells (28). They do, however, abolish IL-2 production, an important cytokine promoting the polarization of Th cells into Th1 phenotype. This regulatory mechanism may be useful for future therapies. The modulation of number/function of T reg lymphocytes could be an approach to control the immunopathology of the disease (40).

PUTATIVE PATHOGENESIS

Despite the fact that the main antigen that triggers sarcoidosis remains unknown, few pathogens are thought to contribute to disease development. For example, mycobacterial protein catalase peroxidase (mKatG) is a tissue antigen and a target of the adaptive immune response in systemic sarcoidosis (41-43) (Fig. 1). Moreover, mKatG is not the only molecule that T cells respond to. A response of sarcoidosis patients' Th1 lymphocytes against mycobacterial superoxide dismutase A (SodA) or early secreted antigenic target 6 (ESAT-6) protein, have also been reported (44). It is notable that unlike the cells from BAL fluid, the peripheral blood mononuclear cells (PBMCs) could not recognize these antigens. However, in a different study it was proven that *M. tuberculosis* antigen 85A can be recognized by Th1 cells from the circulation (45). Furthermore, 4 peptides within antigen 85A have been identified as immunogenic, thus may induce Th1 response in sarcoidosis patients. Despite the confirmed immunopathogenesis of *M. tuberculosis*, it is still not known whether mycobacterial proteins may have an important role in the activation of the immune response in sarcoidosis (45). Other reports suggest that *Propionibacterium acnes* and *Propionibacterium granulosum* may be the causative agents (12). It is well known that *P. acnes* belong to human microbiome and reside mainly in the skin, but could also be present in the lymph nodes. Thus, the current understanding is that multiple exogenous antigens are incriminated in the pathogenesis of sarcoidosis.

Other hypothesis suggests that sarcoidosis is an autoimmune disease. Since no single agent was found present in the granulomas and none to be responsible for triggering the immune response, it is suspected that sarcoidosis develops due to dysfunction in the immune reactions. Previously mentioned activation of TLR-9 supports this hypothesis. However, no particular signaling pathway has been indicated as impaired or inactive during the interaction between DCs, T lymphocytes, and other leukocytes. The defect in immunoregulation may affect either APCs or lymphocytes or both, however, it remains to be elucidated whether the main cause concerns incorrect signal transduction through the immunological synapse between these cells.

microRNAs IN THE IMMUNE RESPONSE

Small, non-coding RNA molecules of about 20 nt in length, known as microRNAs (miRNAs) were shown to regulate expression of 50 – 60% of genes in human genome using RNA interference mechanism. A single miRNA molecule may interfere with up to 100 mRNA molecules (46). These short particles are coded in regions located both within introns of protein-coding fragment (mirtrons) as well as outside of them. Some miRNAs are grouped into so-called clusters - structures that are coordinately transcribed, while others remain as distinct units. Their expression is driven by RNA polymerase II, and maturation is driven by two RNase III enzymes (Droscha and Dicer), that modify primary transcript into final, regulatory molecule. MicroRNAs' binding to 3' untranslated region (UTR) of a transcript may result in decrease of stability by facilitation

of deadenylation or repression of translation, thus a functional protein is not produced (46). It is important to note that these effects do not require the sequence of miRNAs and mRNA to be fully complementary. An RNA-induced silencing complex with a specific miRNA within (miRISC), may block translation in the process called miRNA-mediated mRNA decay, thus controlling functionality of specific cells and tissues (46). Importance of miRNA in embryonic development, cell differentiation and proliferation, as well as in apoptosis and neoplasia has already been described (47-51).

Although, the immune response is highly regulated at the level of transcription, RNA interference plays a significant role in mechanisms of both innate and adaptive immunity (52). The most important molecules in the non-specific response are miR-9, miR-146a and miR-155, as they regulate the activation of myeloid cells. Targets of these miRNAs were proved to down-regulate proteins of Toll-like and interleukin-1 receptor (TIR) signaling pathway. Among them there are: Fas-associated death domain protein, I κ B kinase ϵ (IKK ϵ), receptor interacting serine-threonine kinase 1 (Ripk1), TGF- β activated kinase 1/MAP3K7 binding protein 2 (TAB2), nuclear factor- κ B1 (NF- κ B1), tumor necrosis factor receptor-associated factor 6 (TRAF6) and IL-1 receptor activated kinase-1 (IRAK1) (53-56). What is more, miR-155, miR-223 and the miR-17 – 92 cluster are thought to regulate myeloid cells' proliferation and differentiation (57-59). As for the acquired immune response, RNase III Dicer knockout mice proved that miRNAs are crucial for T and B cell proliferation as well as for development and immunosuppressive activity of Treg lymphocytes (48, 60-64). It is essential to remember that despite a great influence of these molecules onto protein biosynthesis, these post-transcriptional regulators of translation depend on the transcriptional content of the cells.

microRNAs IN SARCOIDOSIS

In the context of sarcoidosis it is important to determine if the abnormalities in immune system are somehow connected to the regulation of translation by miRNA molecules. Of note is that FoxP3, a transcription factor needed for differentiation of T cells into Treg lymphocytes, induces miR-155 expression which, however, does not affect Treg immunosuppressive functions (65, 66). Also, miR-155 was shown to be required for the release of cytokines such as IL-2 and IFN- γ by Th1 cells and in the case of thymic differentiation it down-regulates the suppressor of cytokine signaling 1 (SOCS1), a negative regulator of IL-2R signaling, and regulates cells survival (67) (Fig. 2). Considering the increased number of regulatory lymphocytes in patients' blood and lymph nodes this molecule may be a possible target for future therapies. Interestingly, it was found that miR-155 knock-out animals display a bias towards T helper 2 (Th2) differentiation indicating that miR-155 promotes differentiation into Th1 cells (67, 68). Another molecule miR-31, was shown to inhibit T cell differentiation into T reg lymphocytes by binding to a potential target site within 3' UTR of FoxP3 mRNA and down-regulate this protein synthesis (69) (Fig. 2).

Another key element regulating Treg cells is miR-146a, whose deficiency, contrary to miR-155, results in increased number of these cells, defect in their immunosuppressive function and deregulated IFN- γ response (70). The disruption of immunological tolerance manifests itself in IFN- γ -dependent immune-mediated lesions in many organs, most possibly as an effect of increased expression of STAT1. STAT1 is a key transcription factor in IFN- γ response and a known target of miR-146a (71). One of the reasons for increased expression of STAT1 might be the downregulation of its mRNA degradation - a result of the lack of miR-146a. Also, STAT1 activation in Treg

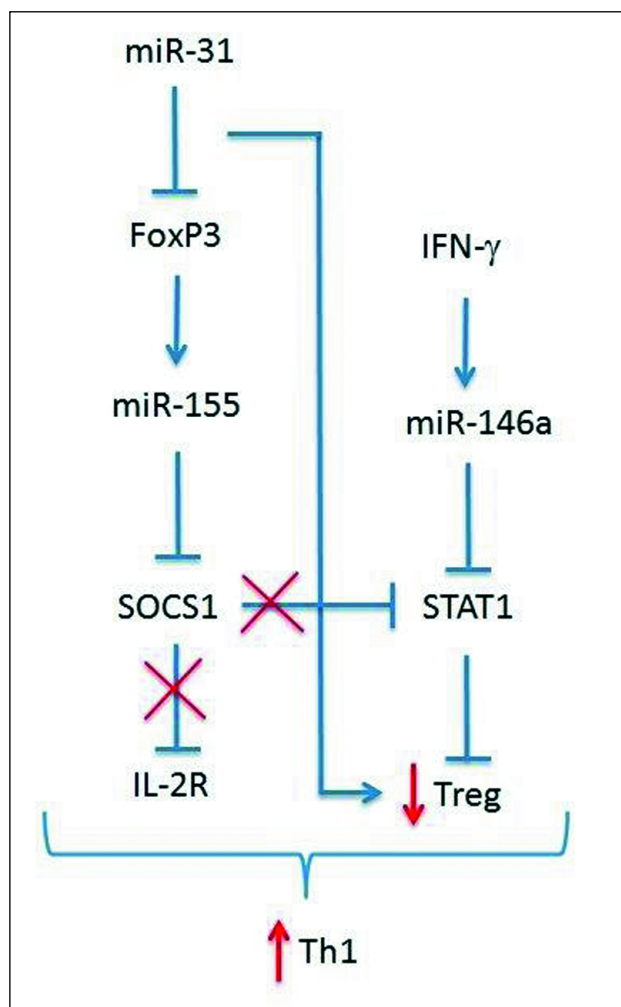


Fig. 2. MicroRNA-mediated regulation of T cell development and function - a flow chart showing the possible involvement of miRNAs in the pathogenesis of sarcoidosis.

FoxP3 - the main regulator of T lymphocytes differentiation into T regulatory cells, which can be blocked by miR-31, induces miR-155 expression, which targets SOCS1, a negative regulator of STAT1 signaling. In contrast - miR-146a, which can be activated by IFN- γ , suppresses STAT1 and reduces the number of Treg cells. SOCS1 is also a negative regulator of IL-2R signaling and its inhibition by up-regulated miR-155 can improve the Th1 cell survival. Consequently, a shift towards Th1 cells can happen in sarcoidosis.

cells with selective ablation of SOCS1, a negative regulator of STAT1, resulted in similar Th1-mediated pathology (65). This leads to the conclusion that an optimal level of STAT1 within the cell is required for proper regulation of Th1 cells by Treg lymphocytes (71). A signaling network between Tregs, Th1 cells, SOCS1, STAT1, IFN- γ , IL-2, miR-155 and miR-146a can be important also in the context of sarcoidosis. Additionally, miR-155 was shown to be one of the microRNAs that is differentially expressed in M1 and M2 macrophages (72), which play also part in the granuloma formation in sarcoidosis.

Lately, Crouser *et al.* conducted a microarray analysis of lung tissue as well PBMCs of sarcoidosis patients and healthy people in order to determine the level of various miRNA molecules (73). As the results show, some regulatory RNA expressions may be lowered or elevated in relation to the control samples, depending on the origin of the cells. Bioinformatic analysis of differentially expressed miRNAs has been performed and molecular targets of identified factors have been predicted. Despite the fact that profiles of miRNA expression in patients' PBMCs and lung tissue do not overlap, in both cases the predicted targets are within TGF- β /WNT pathways (73). It has been observed, that higher TGF- β production correlates with spontaneous regression of sarcoidosis (74). Also, this factor is needed for the survival and expansion of T lymphocytes (75, 76). Interestingly, another study shows that TGF- β increases miR-155 expression, but downregulates IL-2 and IFN- γ in CD4⁺ cells (77). This regulation, in the context of complete inhibition of IL-2 in sarcoidosis patient cells, requires further investigation. Two other papers suggested that pulmonary fibrosis, which is observed in significant percentage of chronic sarcoidosis patients, may be dependent on TGF- β gene variant (78, 79). On the other hand, WNT signaling pathway, which is responsible for T cells development, as well as Treg and DCs activation (80) was shown to be activated in sarcoidosis (81).

Interestingly, a recent report presents a molecular-level comparison of 2 pulmonary inflammatory diseases: sarcoidosis and tuberculosis (82). Cytokine levels in patients serum and miRNA expression in PBMCs were analyzed. Surprisingly, as many mRNA transcripts and miRNA molecules from blood cells were found to be expressed at similar level in both diseases. Therefore, these two pathological conditions seem to share common inflammatory underlying mechanisms. The molecule miR-144, involved in the cellular response to oxidative stress, was shown as the most strongly up-regulated factor in both diseases in comparison to healthy controls (82). While determining the presence of cytokines in sera the authors found out that, in general, tuberculosis is characterized by higher expression of pro-inflammatory cytokines (such as IFN- γ , IL-12) while sarcoidosis sera contained lower concentration of molecules mediating chemotaxis and stimulating granulocytes (Chemokine (C-C motif) ligand 5, Chemokine (C-C motif) ligand 27, stem cell growth factor beta and leukemia inhibitory factor). Also, in both cases transcriptional profiling of blood cells revealed activation of common pro-inflammatory pathways. It was suggested that pulmonary sarcoidosis is the closest matching disease to active pulmonary tuberculosis (82).

Very recently our group has shown that PBMCs isolated from sarcoidosis patients contained significantly higher level of miR-34a than cells obtained from healthy individuals (83). This molecule was previously found to be up-regulated in melanoma cells stimulated with IFN- γ (84), a cytokine shown also to be increased in sarcoidosis patients' sera (83), lymph nodes (85) and BAL fluid (86). MiR-34a was described as a tumor suppressor that inhibits silent information regulator 1 (SIRT1). SIRT1 downregulation leads to an increased level of acetylated p53 and colon cancer cells apoptosis (87). Moreover, inhibition of SIRT1 by miR-34a causes disruption in cellular energy

metabolism and stimulates the NF- κ B-induced inflammatory responses (88). Interestingly, recently it was shown that miR-34a is induced following TCR-induced T cell activation and down-regulates diacylglycerol kinase ζ (DGK ζ) via its seed matches in both coding region and 3' UTR of DGK ζ , resulting in enhanced expression of T cell activation marker CD69 (89).

Although there has been substantial progress in understanding the biochemical and molecular pathways involved in the pathology of sarcoidosis over the past few years, several important questions remain to be answered. First, is there only one key agent leading to the disease progression? Second, what are the genes or their variants that increase susceptibility to develop sarcoidosis and shape the clinical outcome of the individual patient? However, despite the incomplete understanding of disease mechanisms, the diagnostic strategies of sarcoidosis have improved. Determining the causes and populations at risk for the disease would not only improve diagnosis and treatment, but possibly aid in prevention. For that, further research on the molecular mechanisms of the disease is needed. Intracellular gene expression, as well as, cell-to-cell signaling should be studied deeper in order to build an advanced model of etiology and immunopathogenesis. To achieve that, it might be important to investigate the role of miRNAs in regulation of specific cell's transcriptome, such as the role in the immune response and regulatory T cells.

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